Design and production of silica and ORMOSIL nanoparticles for gene delivery

Joana Catarina Capinha de Matos

Under supervision of Maria Clara Henrique Baptista Gonçalves and Gabriel António Amaro Monteiro
Dep. Bioengenharia, IST, Lisbon, Portugal

Abstract

Plasmids (pDNA) are widely used in clinical and non-clinical research as non-viral vectors in gene delivery, being one of the main research areas of nanobiotechnology. However, plasmids are quite inefficient as mediators of gene expression, when compared with viral vectors, due to their degradability by extra- and intracellular nucleases. It is thus important to develop alternative delivery systems able to protect pDNA.

Silica and organically modified silica (ORMOSIL) nanoparticles (NPs) are a promising candidate to gene carriers due to their high biocompatibility. Their versatility for functionalization allows tunable hydrophilic/hydrophobic NPs, with a high payload capacity, giving rise to wide range of biomedical and pharmaceutical applications.

In this work, silica and ORMOSIL NPs were synthesized by a bottom-up approach, a modified LaMer method. The process was optimized in order to obtain NPs with the designed characteristics for an efficient pDNA complexation and further cell transfection.

The bio-conjugation was performed with the pDNA, pVAX1-GFP (3,697 bp). The synthesized NPs were characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS) and zeta potential. Transfection studies were made with Chinese hamster ovary (CHO) cells and its efficiency evaluated by flow cytometry and confocal images. Zebrafish embryos were used to assess cytotoxicity, NPs/complex biodistribution and to evaluate the expression of the green fluorescent protein (eGFP).

Keywords: Silica, ORMOSIL, Nanoparticles, Gene Therapy, Non-viral vectors, Transfection, Zebrafish embryos, Toxicity, biodistribution.

1 Introduction

Gene therapy involves transferring genetic material into a cell, tissue or organ in order to treat or to improve a clinical status. A wide range of gene delivery vectors, viral and non-viral, have been studied and developed over the last decades. Gene delivery is usually performed by viral vectors. However, these delivery systems usually carry disadvantages as their potential immune response, difficult recombination and high cost which have restricted some of their applications.

In order to overcome these limitations, non-viral vectors have been increasingly studied. They have important advantages over viral vectors, due to their low host immunogenicity, and their large capacity for DNA therapeutics. Direct injection of naked DNA is the simplest method of non-viral gene delivery.

However, non-viral delivery systems are quite inefficient as mediators of gene expression when compared with viral vectors, as these molecules are degraded by extra- and intracellular nucleases. Therefore, the lack of efficient and safe gene delivery methods is a critical concern for gene therapy.

The success and effectiveness of gene therapy in in vivo experiments depend on the ability to produce large amounts of pDNA, according to pre-defined specifications, as well as the development of gene de-
livery agents that protect the pDNA and increase pDNA cell uptake. Non-viral gene carriers normally include organic polymers and liposomes [1][2]. However, an ideal method, with high transfection efficiency and relatively safe, both in vitro and in vivo, is yet to be found.

Inorganic NPs (such as hydroxyapatite and silica) present high biocompatibility, stable chemical/physical properties and lack of toxicity, thus they are promising alternatives as gene carrier agents [3].

The sol-gel Stöber method, widely used in the synthesis of SiO$_2$ NPs, allows the production of monodisperse NPs through the hydrolysis and condensation reactions of a mixture of alkoxysilane precursors, such as tetraethoxysilane (TEOS), in mild basic aqueous medium, to create monodisperse, spherical, electrostatically-stabilized particles (or coatings) [4]. Although parameters such as solvent, temperature, base concentration, and water to TEOS ratio can be adjusted to control the particle size (or coating thickness), monodisperse, regular shape SiO$_2$ NPs (<100nm) are difficult to obtain.

Sol-gel synthesized NPs offer many advantages, namely a very high purity level. Their low cytotoxicity makes them particularly interesting for medical applications.

Several attempts have been made to develop procedures for modifying the surfaces chemistry (functionalization procedures), in order to increase their biocompatibility, improve its resistance to enzymatic action and internalization efficiency. ORMOSIL NPs have advantages over SiO$_2$ NPs, since the presence of non-hydrolysable organic groups allows tuning the surface hydrophilic/hydrophobic character. The ORMOSIL NPs can be easily loaded with biomolecules such as proteins and drugs, among others.

Aminopropyltriethoxylane (APTES) is a commonly used alkoxide precursor for the in situ surface functionalization of polymeric vectors used for gene delivery. The amino group will electrostatically interact with proteins, enhancing their adsorption.

It has been shown that SiO$_2$ NPs, functionalized with amino groups, bind and protect pDNA from enzymatic digestion allowing cell transfection in vitro. In the present work, silica and ORMOSIL NPs were synthesized with a modified LaMer method, based in Huang et al. [5] work.

2 Materials and Methods

2.1 Silica and ORMOSIL NPs Synthesis

Two different procedures were followed in the NPs synthesis: the modified Stöber method, described in [4], and a novel adaptation of the modified LaMer method, described in [5].

The silica and ORMOSIL NPs were synthesized using ammonia hydroxide solution (NH$_4$OH, 28-30%), absolute ethanol (EtOH, 99.5%), aqueous sodium silicate solution (SSS, Na$_2$O.SiO$_2$, 27% wt.% SiO$_2$), tetraethylorthosilicate (TEOS, reagent grade 98%) and aminopropyltriethoxysilane (APTES, ≥98%). The washings, carried out after NPs reaching a intended size, were performed using ethanol 70% and bi-distilled water (Conductivity at 0-2µS/cm, pH at 5.8-6.5).

ORMOSIL NPs synthesis by the modified Stöber method

The synthesis of the monodisperse NPs was performed following the modified Stöber method, allowing the synthesis of amino-NPs with diameters smaller than 300nm at room temperature in less than one hour.

The synthesis of these amino-NPs is achieved using aqueous SSS as a nucleating agent and a combination of an inorganic precursor (TEOS) and an organic precursor (APTES). Initially, a dilution of aqueous sodium silicate solution with bi-distilled water in a ratio of 500:20 was made and placed in ultrasonication for 20 minutes. A volume of 280µL of this solution was then diluted in 25mL of ethanol and the resulting solution placed under magnetic stirring for 15 minutes. A mixture of 12.6mL of ethanol and 28mL of ammonia solution was then added to the suspension and stirred for 15 minutes. After this time, a mixture of 1.5mL of inorganic/organic silica precursor with TEOS:APTES-9:1 ratio was added to the suspension, followed by 30 minutes of stirring, before centrifuge and washing twice with ethanol 70% and bi-distilled water. After synthesis, the amino-ORMOSIL NPs were dried in the incubator at 40°C for a period of 72h and weighed on an analytical balance (Mettler Toledo - Analytical Balance XS105).
ORMOSIL NPs synthesis by the modified LaMer method

Silica and ORMOSIL NPs (TEOS:APTES(T:A) - 10:0, 9:1, 7:3 and 3:7) synthesis starts with the hydrolysis of the alkoxide precursors, resulting in the formation of the silanol groups. Then silanols react with other silanols or with siloxanes, allowing the NPs growth. Briefly, the synthesis of silica and ORMOSIL NPs was performed mixing 44.7mL of ethanol, 1.15mL of ammonia solution and 2.25mL of bi-distilled water. The resulting solution was then placed under magnetic stirring at 50°C. After temperature stabilization, 1.9mL of precursors (TEOS and mixtures of TEOS and APTES with desired molar ratios) were quickly added, under vigorously magnetic stirring.

Finally, the solution was left in magnetic stirring at 50°C between 10 to 30 minutes since, depending on the precursors ratios, the growth time is variable. The NPs growth was freeze by fast cooling the suspension to room temperature immediately followed by centrifuge (8,000rpm, 20°C, 15 min). Finally, the suspension was washed twice with ethanol 70% and bi-distilled water. After synthesis, the silica and the ORMOSIL NPs were dried in the incubator at 40°C for a period of 72h and weighed on an analytical balance (Mettler Toledo - Analytical Balance XS105).

Size and Zeta Potential determination

To analyze the samples by TEM, a drop of the suspension was placed on a copper grid and dried at room temperature. TEM Hitachi H-8100 model was used and the micrographs were obtained using an applied tension of 200kV.

Determination of silica and amino-ORMOSIL NPs hydrodynamic diameter (HD) and zeta potential (ζ) was performed using the Zetasizer Nano Instrument (Malvern, UK). Samples with 1mL of volume were used and 1:10 dilutions were prepared before the measurements.

2.2 ORMOSIL NPs labeling

In order to visualize the path of the NPs or pDNA:ORMOSIL-NPs complexes during the in vitro and in vivo assays, whose results were analyzed through fluorescence microscopy and confocal images, the NPs were previously labeled with fluorescein-isothiocyanate (FITC) or rhodamine fluorescent dyes.

The ORMOSIL NPs (TEOS:APTES - 7:3 and 3:7) were directly incubated with the fluorescein-isothiocyanate (FITC). The fluorescent dye was initially diluted twice from a stock solution at 800µM concentrated, with borate buffer 100mM, pH 9.

In case of labeling with rhodamine dye, the ORMOSIL NPs were directly incubated, diluted in a ratio of 20:1,000 with borate buffer 100mM, pH 9.

For both cases, the incubation was performed for 30 minutes at room temperature in the dark. To ensure the correct labeling of the NPs, a volume of 1.5µL of labeled NPs was placed on a plate and brought into a drying oven at 37°C and leave for 30 minutes to dry. Finally, it was observed under a fluorescence microscope (Leica DMLB). Unlabeled ORMOSIL NPs were used as control.

2.3 Bio-Conjugation of pDNA with ORMOSIL NPs

The pDNA used for the bio-conjugation (complexation) with the ORMOSIL NPs was pVAX1-GFP (3,697bp). The plasmid was prepared growing Escherichia coli DH5α cells in shake-flasks with 5mL of LB Broth medium and 30µg/ml kanamycin. The shake-flasks were left to incubate at 37°C overnight in an orbital shaker (250rpm). The extraction and isolation of plasmid was performed using the procedure described in High Pure Plasmid Isolation Kit (Roche, Germany) [6]. The bio-conjugation of the ORMOSIL NPs and pDNA (pDNA:ORMOSIL-NPs complexes) was performed by direct incubation of pDNA and ORMOSIL NPs in different ratios. The mixture was placed in the vortex mixer for 30 seconds followed by a period of 30 minutes at room temperature. The bio-conjugation between the pDNA and ORMOSIL NPs was analyzed using the electrophoretic mobility of pDNA in 1% agarose gel (Seakem LE Agarose). Several weight ratios of pDNA to ORMOSIL NPs were tested.

2.4 In Vitro Studies: Transfection

Transfection studies were performed using CHO cells. These cells were grown in complete Dul-
becco’s modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and antibiotics, 1% PenStrep. The cells were grown in 75cm² culture flasks (Incubator 37°C, 5% CO₂) up to confluence, trypsinised and seeded in 24-well/8-well culture plates. Cells were incubated and then transfected with pDNA:NPs complexes. Transfection was performed following the Protocol of Transfection using Lipofectamine 2000 [7] (1µg of pDNA plus 1.5µL of Lipofectamine in 100µL of medium per well). Lipofectamine 2000 was used as control. The amount of pDNA in each well was kept constant (1µg) only varying the amount of complexed amino-NPs. Medium containing only pDNA (1µg/well) was also used as control. The transfection period was 2/3/6 hours and the cells remained in contact with the transfection solution during this time. After this period the medium containing the transfection solution (in the case of the negative control, the medium containing only pDNA) was removed and replaced by fresh complete media containing 10% FBS and 1% PenStrep. Cells (transfected and non-transfected) were then left incubating at 37°C with 5% CO₂ for 24h/48h.

The transfection efficiency was evaluated using flow cytometry analysis after 24h/48h of transgene expression and confocal images.

2.5 Zebrafish embryos

The zebrafish (Danio rerio) model presents many features that make it well suited for studies of nanomedical applications, like a short generation time and a large hatch size. Zebrafish also presents a rapid embryogenesis. This rapid development and the optical transparency of the embryos allow the monitoring of organ development.

Zebrafish adults are maintained in carbon-filtered water complemented with salt “Instant Ocean Synthetic Sea Salt” (Spectrum Brands, USA), at 27 ± 1°C and under a 14:10h light:dark photoperiod cycle. Conductivity is kept at 750±50 µS/cm, pH at 7.5±0.5 and the saturation of dissolved oxygen >95%. The test solutions used in the zebrafish assays were made with the above described culture water. Zebrafish eggs were collected immediately after natural mating and were rinsed with water and checked under a stereomicroscope (Stereoscopic Zoom Microscope - SMZ 1500). Animal husbandry at the Department of Biology, University of Aveiro, followed the Portuguese law for animal experiments.

2.5.1 In vivo studies: FET studies with ORMOSIL NPs and complexes

The assay was based on the OECD guideline on Fish Embryo Toxicity Test (FET). Sixty eggs were used per treatment and distributed in 24-well microplates. The eggs were placed in each well individually with 2mL of test solution. Five treatments were used: i) control, only culture water; ii) T:A-9:1 NPs; iii) T:A-7:3 NPs; iv) T:A-3:7 NPs; and v) complexes (T:A-3:7 NPs). The amount of NPs used corresponded to the maximum concentration tested for complexation with pDNA. The ORMOSIL NPs were sonicated for 45 minutes before use. After placing the eggs in the different treatment solutions, the microplates were taken into a chamber which is at a constant temperature of 25±2°C. During the test, the embryos were maintained in this chamber and observed daily over 4 days with the stereomicroscope.

2.5.2 In Vivo Studies: ORMOSIL NPs Microinjections

In vivo assays were performed in two ways: microinjecting 48hours post fertilization (hpf) embryos with NPs/complexes and microinjecting one cell eggs with only pDNA:NPs complexes.

Microinjection of 48hpf Zebrafish Embryos

The D. rerio embryos were divided in 5 different groups to test different conditions: i) control, microinjected with PBS only, ii) microinjected with ORMOSIL NPs (T:A - 7:3), iii) microinjected with ORMOSIL NPs (T:A - 3:7), iv) microinjected with complexes(T:A - 7:3) and v) microinjected with complexes(T:A - 3:7). With the exception of complexes samples, fluorescein-isothiocyanate-dextran (FITC-Dextran) was added to the samples to allow the visualization of the particles bio-distribution, in comparison with the control. Phenol red solution (0.5% in DPBS) was added to the samples prepared for microinjection, to confirm if the embryo was well injected. FITC-Dextran was not added to the com-
plexes samples, since if the gene delivery is successful, the eGFP protein expression will occur.

**Microinjection of Zebrafish one cell Eggs**

The main objective of this assay was to verify the eGFP expression on zebrafish embryos. The *D. rerio* one cell eggs were divided in 4 groups to test different conditions. Two of those groups were microinjected with pDNA complexed with T:A(7:3) NPs and the other 2 with pDNA complexed with T:A(3:7) NPs. In both cases, the tested complexes proportions were 1:60 and 1:30. The preparation of the suspension to be microinjected was performed adding 1 µL Phenol Red Solution (0.5% in DPBS) (to confirm if the eggs were well microinjected), 2 µL of KCl buffer (0.4M), and 7 µL of the pDNA:NPs complexes. As previously mentioned the eggs were collected immediately after natural mating, and in this case, they were immediately microinjected for each tested condition.

### 3 Results and Discussion

#### 3.1 Characterization of Silica and ORMOSIL NPs

ORMOSIL NPs (T:A - 9:1) were synthesized following the modified Stöber method described in Colaço et al. [4]. SSS was then used as seed for nanoparticle growth. The number of NPs produced depends on the number of nuclei available, i.e., the initial SSS concentration. These nuclei have silanol groups at their ends which will bind to the silanol groups formed by hydrolysis in the sol-gel process of the alkoxides precursors, TEOS (inorganic) and APTES (organic), to form siloxanes. After this, the silanols react with other silanols or with siloxanes thus allowing the amino-NPs growth. Their growth can also be affected by monovalent cations present in the SSS suspension. The NPs size as well as their morphology is affected by the presence of organic groups [4]. The absolute yield of the synthesis was obtained by drying and weighing the NPs. Figure 1 shows the images obtained by TEM.

![Figure 1: TEM images ORMOSIL NPs (TEOS:APTES - 9:1).](image)

The average diameter (TEMD) of the NPs is 255nm and they present a high degree of monodispersity, i.e., a small standard deviation in the particle size. The average diameter of the particles was obtained using the ruler tool from Adobe Photoshop CS5, after proper calibration.

On the other hand, silica and amino functionalized ORMOSIL NPs with different T:A (10:0, 9:1, 7:3 and 3:7) ratios were synthesized through an adapted protocol based on the methodology described by [5]. The size, and therefore the number, of synthesized silica and ORMOSIL NPs depend on the temperature and reaction time. The size, size distribution and morphology are also affected by the presence of organic groups. Figure 2 shows the images obtained by TEM.

![Figure 2: TEM images: (A) Silica NPs - 2h of reaction time; (B) ORMOSIL NPs (T:A - 9:1) - 2h of reaction time; (C) ORMOSIL NPs (T:A - 9:1) - 1h of reaction time; (D) ORMOSIL NPs (T:A - 7:3) - 30min of reaction time; (E) ORMOSIL NPs (T:A - 7:3) - 15min of reaction time](image)

NPs diameters in the range 80-180 nm were obtained, with a fair size distribution, which is a good combination for gene delivery. Particle size, and the number of particles synthesized per batch slightly
decrease with reaction time. The static diameter (TEMD) values were obtained from TEM images using Adobe Photoshop CS5. As shown in the results, a moderate monodispersity was obtained.

In both process of synthesis, the yield was obtained by weighting the dried NPs. However, this procedure has an error hard to quantify. After the synthesis, the NPs were washed four times in order to interrupt the NPs growth, which may lead to an unquantified mass loss.

The size of the NPs synthesized through the novel LaMer method was also characterized by DLS, obtaining the hydrodynamic diameter (HD), which is significantly higher than the TEMD. This difference can be explained by the hydrophillic character of silica and amino groups. Due to its hydrophilicility, the water can penetrate the NPs pores by capillary effects and by the silica/ORMOSIL matrix compliance, which will increase the obtained HD. For this reason it was expected that the HD values were higher than those of TEMD, however such a large difference can also be due to the fact that the particles begin to aggregate. This is corroborated by the different PdI (polydispersity index) values which are higher on solutions with aggregated particles.

The zeta potential, measured only for the ORMOSIL NPs synthesized by the novel LaMer method, shows a great dependence on the dispersion medium. The suspension is considered stable when the absolute value of the potential is larger than 30mV. On the other hand, low positive charge densities are preferred since they indicate a reduced cytotoxicity. The positively charged NPs can combine with the negatively charged pDNA by electrostatic interaction, this combination is less likely to occur with negatively charged NPs.

3.2 ORMOSIL NPs labeled with fluorescent dyes

To ensure the correct labeling of the NPs, a small volume of labeled NPs was dried and observed at fluorescence microscope (Leica DMLB). Images were acquired with a digital camera (Olympus DP 10).

Fluorescein-isothiocyanate (FITC) successfully binds with both T:A(7:3) NPs and T:A(3:7) NPs. However, that binding is more efficient for the T:A(3:7)NPs, which have a higher amine percentage in their composition. This is an expected result, since it is known that amine groups have a high affinity to connect with the FITC.

Rhodamine also successfully binds with both T:A(7:3) NPs and T:A(3:7) NPs. In this case, it is also visible a more efficient binding to the T:A(3:7) NPs, due the same reasons given in the case of labeling with FITC.

3.3 Gel electrophoresis analysis

To analyze the bio-conjugation between pDNA and the amino functionalized ORMOSIL NPs, different mass ratios of pDNA:ORMOSIL-NPS were tested for the three precursors ratio, using the electrophoretic mobility of pDNA in agarose gel. The complexation between the pDNA and the ORMOSIL NPs is due to the electrostatic bonds established between the cationic amino groups present in NPs and the anionic phosphate groups of the plasmid.

The efficiency of the complexation can be assessed by agarose gel electrophoresis, since an efficient complexation leads to pDNA immobilization inside the wells of the agarose gel, as NPs do not enter the gel.

In case of the complexes formed with the ORMOSIL NPs synthesized by the Stöber method, is visible in figure[②] that the quenching of the fluorescence, when compared with the control pDNA, is clearly visible. This suggests an interaction between the plasmids and the amino-NPs, it can thus be concluded that complexation occurs in all tested proportions. However the pDNA is not fully complexed, since the immobilization inside the wells is not total.
Figure 3: Agarose gel electrophoresis of plasmid DNA free and complexed with amino-ORMOSIL NPs: Lane 1, NZY Ladder III; Lane 2, pDNA free; Lane 3, pDNA:NPs - 1:350; Lane 4, pDNA:NPs - 1:175; Lane 5, pDNA:NPs - 1:88.

On the other hand, in case of the complexes formed by the ORMOSIL NPs (T:A - 9:1, 7:3 and 3:7) synthesized by the novel LaMer model, Figure 4 shows that the quenching of the fluorescence, when compared with the control pDNA, is visible. This proves interaction between the plasmids and the ORMOSIL NPs, over the tested precursors ratios. However, complexes formed by ORMOSIL NPs (T:A - 7:3 and 3:7) do not show full complexation in the 2 last tested ratios, since the immobilization of the pDNA in the wells was not complete.

Figure 4: Agarose gel electrophoresis of plasmid DNA free and complexed with ORMOSIL NPs. (A) Agarose gel electrophoresis correspondent to ORMOSIL NPs – T:A (9:1): Lane 1, Ladder NY; Lane 2, pDNA free; Lane 3, 4, 5, 6 and 7 pDNA:NPs – 1:350, 1:100, 1:80, 1:40 and 1:20. (B) Agarose gel electrophoresis correspondent to ORMOSIL NPs – T:A (7:3 and 3:7): Lane 1, Ladder; Lane 2, pDNA free; Lane 3, 4, 5 and 6 pDNA:NPs(7:3) – 1:60, 1:30, 1:10 and 1:5; Lane 7, 8, 9 and 10 pDNA:NPs(3:7) – 1:60, 1:30, 1:10 and 1:5.

Thus, it can be concluded by the presented results that the synthesized NPs and especially the ones which were synthesized by the novel LaMer method, show a great bio-conjugation with pDNA. This is an important feature considering the possibility of the ORMOSIL NPs being used as gene carriers.

3.4 DLS and Zeta Potential characterization of the complexes using ORMOSIL NPs synthesized by the novel LaMer Method

A brief characterization of the complexes was performed by DLS and zeta potential, in order to analyze possible differences in the behavior of the NPs when they are alone or complexed with pDNA.

It is noted that the average HD is smaller when the particles are complexed with the pDNA than when they are alone in the suspension. Moreover it can be seen that, in the complexes, the obtained size appears to be proportional to the amount of NPs in solution. This can be explained by the decrease in hydrophilicity shown by the silica/ORMOSIL NPs, due to their interaction with pDNA. This fact will prevent the entrance of the solvent/water in the pores of the NPs which will reduce the HD values.

The presented PdI values and the obtained correlogram show that the formation of agglomerates is greater in the case of complexes than in case of the particles without the pDNA. Measurements of zeta potential were also made. The obtained results show that the ORMOSIL NPs potential is positive which is an important feature, since they have to bind to the negatively charged pDNA. On the other hand, the complexes present a negative potential which might result from the fact that the negative charges of pDNA stayed in the particles surface.

From these results it is important to note that the negative charge of the complexes is likely to prevent or hinder the interaction of the complexes with the cell membrane, which will directly influence the transfection rate.

3.5 In vitro studies analyzed by flow cytometry

ORMOSIL NPs synthesized by the modified Stöber method

In the present study the surface of SiO₂ NPs was modified by introducing amino groups and their ability to deliver the eGFP gene into CHO cells evaluated. The transfection study was performed consid-
ering the pDNA:NPs mass ratios: 1:350, 1:175 and 1:88. In this transfection experiment, the culture flasks showed 80% of cell confluence. The duration of transfection, known as the time in which the NPs are in direct contact with the cells, was 2h and the post transfection incubation was 24h. This incubation time corresponds to the time between transfection and measurement of the GFP fluorescence using flow cytometry.

For the tested ratios 1:350 and 1:175, the number of events was below the accepted value (<5000), it was thus not possible to consider the percentage of transfection in these cases. Figure 3.15 shows the transfection results obtained for the positive control (Lipofectamine 2000) and the pDNA:NPs 1:88 ratio. In the 1:88 ratio, the transfection percentage was approximately zero. However, the percentage of transfection achieved in the positive control is approximately three times smaller than expected. The transfection studies are thus inconclusive.

**ORMOSIL NPs synthesized by the modified LaMer method**

On the first transfection assay, performed with the ORMOSIL NPs synthesized by the novel LaMer method, the parameters were identical to the ones in the assay using the NPs synthesized by the Stöber method. The culture flasks showed 80% of cell confluence. The duration of transfection, was 2h and the post transfection incubation was 24h. The transfection study was performed considering the pDNA:NPs(T:A-9:1) mass ratios: 1:350, 1:175 and 1:88.

For the three different tested ratios, the transfection percentage was approximately zero. However, the percentage of transfection achieved in the positive control is also lower than expected, presenting a value approximately three times smaller than usual which makes the assay inconclusive. In the second and the third experiments performed, the culture flasks showed approximately 80% of cell confluence. The duration of transfection was maintained, 2h and two different post transfection incubation times were tested, 24h and 48h. The transfection assay was performed considering the pDNA:NPS(T:A-9:1) mass ratios: 1:88, 1:66, 1:44 and 1:22 for the second experiment and 1:44, 1:22, 1:11 and 1:5 for the third experiment.

The rate of transfection obtained for the complexes tested in both assays is void or approximately null. However, the percentage of transfection obtained for the positive control remains approximately 3 times less than expected which again makes the assay inconclusive. Two more experiments were performed. The main objective was to test different transfection times, 3h and 6h, respectively and also different ORMOSIL NPs, T:A ratios (7:3 and 3:7). However, the cell culture when transferred to the 24-well tissue culture plates and during the assay, began to present some issues relating their normal growth, even losing their usual elongated shape. The results obtained do not allow any treatment since the number of events was below the accepted value (<5000), it was thus not possible to consider the percentage of transfection in these cases. The problem was detected in all the conditions tested, including the positive and negative control. In conclusion, from the assays that were possible to analyze, the transfection percentage for all the complexes tested, was practically null. This is not an encouraging result regarding a possible use of the produced NPs as gene carriers. However, the complexes used as positive control (pDNA plus lipofectamine 2000) also showed a substantially lower transfection rate than what is expected. Thus, all the assays performed are inconclusive.

### 3.6 *In vitro* studies analyzed by confocal microscopy

Only complexes formed by ORMOSIL NPs synthesized by the modified LaMer model were used for these assays.

The ORMOSIL NPs were labeled with the fluorescent dye rhodamine and complexed to the pDNA which will express the eGFP upon the arrival to the nucleus (transfection). The transfection experiment was performed using different pDNA:ORMOSIL-NPs mass ratios complexes. Their uptake to the cell and the occurrence of transfection was analyzed by CLSM images. The images were obtained with a Leica TCS SP5 laser scanning microscope.

Figure 5 shows the pDNA:ORMOSIL-NPs complexes inside the cells, confirming their internaliza-
In order to confirm that the pDNA delivery is functional, the expression of eGFP was verified by transfection of CHO cells. Figure 6 shows that eGFP was transfected successfully using the ORMOSIL NPs (TEOS:APTES - 7:3) as delivery vector.

**Figure 6:** Confocal fluorescence image of CHO cells transfected with pDNA:NPs complexes (ORMOSIL NPS - T:A(7:3)).

### 3.7 Fish Embryo Toxicity Assay

In the present study, fertilized zebrafish eggs were exposed for 120 hours to different ORMOSIL NPs and pDNA:ORMOSIL-NPs complexes to observe their effect in the embryos development, analyzing parameters such as mortality, delays or malformations during the embryos growth.

The results obtained and the images collected by the stereoscope zoom microscope, allow to conclude that the NPs as well the complexes tested do not present toxic effects. The mortality rate is negligible on all assays exhibiting no differences between the control and the treatment groups. The FET test was performed with 60 biological replicates.

### 3.8 In vivo Studies: Zebrafish embryos assays

#### 3.8.1 Microinjection of NPs/complexes in 48hpf Zebrafish Embryos

For *in vivo* studies, 20 Zebrafish embryos were microinjected for each tested condition. To check if the NPs or complexes have a harmful effect on the development of embryos, these were observed during 120hpf. The results obtained and the images collected by both fluorescence microscope and stereoscopic zoom microscope, allowed to conclude that the NPs, as well as the complexes, do not show any cytotoxicity. Regarding the mortality rate, no differences were verified between the control and the microinjected embryos.

The analysis of the NPs distribution in Zebrafish embryos was performed by injecting ORMOSIL NPs conjugated with FITC-dextran, in 48hpf embryos. Although, the images obtained are not very clear, but it is possible to notice differences between the fluorescence of the control and the embryos injected with ORMOSIL NPs. On the controls, the FITC-dextran fluorescence is uniformly distributed over the entire embryo. On the other hand, on the embryos injected with NPs, the FITC-dextran will bind to the NPs, which concentrate in certain areas. Thus, the observed fluorescence is not uniformly distributed.

The results concerning the expression of the eGFP in microinjected embryos with pDNA:ORMOSIL-NPs complexes were not entirely conclusive, since no fluorescence was easily observed. However, close observation of embryos injected with the complexes, reveal some differences, when compared with the control. The *in vivo* assays were performed with 20 biological replicates and two technical replicates, for each tested condition.

#### 3.8.2 Microinjection of complexes in Zebrafish one cell eggs

This last assay was performed to test the eGFP expression, since the results from the injection on 24hpf embryos was not fully conclusive. After being collected, the zebrafish eggs were immediately injected with the test suspensions. The microinjection was performed in the yolk and the eGFP expression was
observed after 24 hours. The embryos developed normally, not presenting delays or malformations but, once again, no fluorescence due to eGFP expression was observed. This assay was performed with 20 biological replicates for each condition tested.

4 Conclusion

This work presented two different procedures to synthesized silica and ORMOSIL NPs. Firstly the modified Stöber method described, in Colaço et al. [4], was used to synthesize amino-ORMOSIL NPs with T:A ratio 9:1. The obtained NPs have diameters in range of 210–290nm, presenting a fair monodispersity as shown in TEM images. However, the size of the obtained amino NPs was not satisfactory for the intended application, gene delivery. The agarose gel electrophoresis, which analyses the complexation efficacy, shows that the pDNA complexes with the amino-ORMOSIL NPs. However, none of the tested pDNA:NPs ratios achieved full complexation. In vitro studies were also performed with these NPs. However, since the transfection percentage observed in the positive control was much smaller than the expected, it is not possible to draw any conclusion from these results.

The second method is a novel modified sol-gel LaMer method. This procedure was adapted in order to introduce non-hydrolysable amino groups, starting with different TEOS:APTES precursors ratio. NPs diameters in the range 80-180nm were obtained, with a fair size distribution, satisfactory for the intended application, gene delivery. The agarose gel electrophoresis shows that complete complexation occurs between the pDNA and the different synthesized amino functionalized ORMOSIL NPs. The performed in vitro tests confirmed that pDNA:ORMOSIL-NPs complexes are able to penetrate the cell membrane, through transfection. However, the transfection assays analyzed by flow cytometry were inconclusive.

Finally, the in vivo tests, performed with amino-ORMOSIL NPs synthesized by the modified LaMer method, allows us to conclude that their cytotoxicity is negligible, due to the low mortality rate and lack of morphological changes in Zebrafish larvae injected with the amino NPs. The transfection studies performed by microinjection of pDNA:ORMOSIL-NPs complexes on zebrafish embryos were inconclusive.

References